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PATENT- OG VAREMÆRKESTYRELSEN

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PHOSPHOLIPASE

FIELD OF THE INVENTION

The present invention relates to a phospholipase, methods of using and producing it, as well as a nucleic acid sequence encoding it.

BACKGROUND OF THE INVENTION

Phospholipases are known to be useful, e.g. in baking and oil degumming.

A phospholipase from *Verticillium dahliae* is disclosed in COGEME Phytopathogenic Fungi and Oomycete EST Database, Unisequence ID: VD0100C34.

Soragni et al., The EMBO Journal, 20 (2001), 5079-5090, discloses a new group of phospholipases defined by organism source, active site sequence similarity and cysteine residue conservation. They name the group "fungal/bacterial group XIII PLA2".

US 6399121 discloses the use of phospholipase in cheese making.

It is an object of the present invention to provide polypeptides having phospholipase activity and polynucleotides encoding the polypeptides. A further object of the present invention is to provide a method for making cheese.

SUMMARY OF THE INVENTION

The inventors discovered and isolated a gene encoding a novel phospholipase from *Fusarium venenatum* A3/5, which was originally deposited as *Fusarium graminearum* ATCC 20334 and recently reclassified as *Fusarium venenatum* by Yoder and Christianson, 1998, Fungal Genetics and Biology 23: 62-80; and O'Donnell et al., 1998, Fungal Genetics and Biology 23: 57-67. The phospholipase belongs to the fungal/bacterial group XIII PLA2 as defined by Soragni et al., The EMBO Journal, 20 (2001), 5079-5090. The inventors also cloned the novel phospholipase encoding gene into an *E. coli* strain, and used the cloned gene to make a construct for expressing the *Fusarium* phospholipase gene in *Aspergillus oryzae*. The inventors transformed *Aspergillus oryzae* with this construct, and isolated the phospholipase from transformed *Aspergillus* cells.

Accordingly, the invention provides a phospholipase which comprises:

a) a polypeptide encoded by the phospholipase encoding part of the DNA sequence cloned into a plasmid present in *Escherichia coli* deposit number DSM 15442; or

b) a polypeptide comprising the amino acid sequence of amino acids 29 to 149 of SEQ ID NO: 2, or an amino acid sequence which can be obtained there from by substitution, deletion, and/or insertion of one or more amino acids;

or

c) an analogue of the polypeptide defined in (a) or (b) which:

i) has at least 80 % identity with said polypeptide, or

ii) is immunologically reactive with an antibody raised against said polypeptide in purified form, or

iii) is an allelic variant of said polypeptide;

or

d) a polypeptide which is encoded by a nucleic acid sequence which hybridizes under medium stringency conditions with a complementary strand of the nucleic acid sequence of nucleic acids 133 to 495 of SEQ ID NO: 1 encoding the mature polypeptide or a subsequence thereof having at least 100 nucleotides.

Finally, the phospholipase of the invention may be a polypeptide which is encoded by a nucleic acid sequence which comprises:

a) the partial DNA sequence encoding a mature phospholipase cloned into a plasmid present in *Escherichia coli* DSM 15442,

b) the partial DNA sequence encoding a mature phospholipase of nucleic acids 133 to 495 of SEQ ID NO: 1,

c) an analogue of the sequence defined in a) or b) which encodes a phospholipase and

i) has at least 80 % identity with said DNA sequence, or

ii) hybridizes at high stringency with a complementary strand of said DNA sequence or a subsequence thereof having at least 100 nucleotides,

iii) is an allelic variant thereof, or

d) a complementary strand of a), b) or c).

Other aspects of the invention provide a recombinant expression vector comprising the DNA sequence, and a cell transformed with the DNA sequence or the recombinant expression vector.

In further aspects the invention relates to a process for producing cheese comprising adding a phospholipase to a dairy composition and producing cheese from the dairy composition, wherein the phospholipase is selected from the group of fungal/bacterial group XIII PLA2 phospholipases.

DETAILED DESCRIPTION OF THE INVENTION

Genomic DNA source

A phospholipase of the invention may be derived from a strain of *Fusarium*, particularly *F. venenatum*, using probes designed on the basis of the DNA sequences in this specification.

A strain of *Escherichia coli* containing a gene encoding the phospholipase was deposited by the inventors under the terms of the Budapest Treaty with the DSMZ - Deutsche Sammlung von Microorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig DE, Germany. The deposit date was 12 February 2003, and the accession number was DSM 15442.

Properties of phospholipase

The phospholipase has phospholipase A activity and is able to hydrolyze lecithin by releasing fatty acid.

Phospholipase activity

Phospholipase activity can be expressed in LEU determined as follows:

Lecithin is hydrolyzed under constant pH and temperature, and the phospholipase activity is determined as the rate of titrant (0.1N NaOH) consumption during neutralization of the liberated fatty acid.

The substrate is soy lecithin (L-α-Phosphotidyl-Choline), and the conditions are pH 8.0, 40.0°C, reaction time 2 min. The unit is defined relative to a standard.

Recombinant expression vector

The expression vector of the invention typically includes control sequences functioning as a promoter, a translation initiation signal, and, optionally, a selectable marker, a transcription terminator, a repressor gene or various activator genes. The vector may be an autonomously replicating vector, or it may be integrated into the host cell genome.

Production by cultivation of transformant

The phospholipase of the invention may be produced by transforming a suitable host cell with a DNA sequence encoding the phospholipase, cultivating the transformed organism under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

The host organism is preferably a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell, such as a strain of *Aspergillus*, *Fusarium*, *Trichoderma* or *Saccharomyces*, particularly *A. niger*, *A. oryzae*, *F. venenatum*, *F. sambucinum*, *F. cerealis* or *S. cerevisiae*, e.g. a glucoamylase-producing strain of *A. niger* such as those described in US 3677902 or a mutant thereof. The production of the phospholipase in such host organisms may be done by the general methods described in EP 238,023 (Novo Nordisk), WO 96/00787 (Novo Nordisk) or EP 244,234 (Alko).

Hybridization

Hybridization is used to indicate that a given DNA sequence is analogous to a nucleotide probe corresponding to a DNA sequence of the invention. Hybridization conditions are described in detail below.

Suitable conditions for determining hybridization between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5 x SSC (standard saline citrate) for 10 min, and prehybridization of the filter in a solution of 5 x SSC (Sambrook et al. 1989), 5 x Denhardt's solution (Sambrook et al. 1989), 0.5 % SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook et al. 1989), followed by hybridization in the same solution containing a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132:6-13), ^{32}P-dCTP-labeled (specific activity > 1 x 10^9 cpm/µg) probe for 12 hours at approx. 45°C. The filter is then washed two times for 30 minutes in 2 x SSC, 0.5 % SDS at a temperature of at least 55°C, more preferably at least 60°C, more preferably at least 65°C, even more preferably at least 70°C, especially at least 75°C.

Molecules to which the oligonucleotide probe hybridizes under these conditions are detected using x-ray film.

Alignment and homology

In a first embodiment, the present invention relates to polypeptides having phospholipase activity and where the polypeptides comprises, preferably consists of, an amino acid sequence which has a degree of identity to amino acids 29 to 149 of SEQ ID NO:2 (i.e., the mature polypeptide) of at least 80%, such as at least 85%, even more preferably at least 90%, most preferably at least 95%, e.g. at least 96%, such as at least 97%, and even most preferably at least 98%, such as at least 99%.

Preferably, the polypeptides of the present invention comprise the amino acid sequence of SEQ ID NO: 2; an allelic variant thereof; or a fragment thereof that has phospholipase activity. In another preferred embodiment, the polypeptide of the present invention com-

prises amino acids 29 to 149 of SEQ ID NO:2. In a further preferred embodiment, the polypeptide consists of amino acids 29 to 149 of SEQ ID NO: 2.

The present invention also relates to a polynucleotide comprising, preferably consisting of, a nucleotide sequence which has at least 80% identity with nucleotides 133 to 495 of SEQ ID NO:1. Preferably, the nucleotide sequence has at least 85% identity, such as at least 90% identity, more preferably at least 95% identity, such as at least 96% identity, e.g. at least 97% identity, even more preferably at least 98% identity, such as at least 99% with nucleotides 133 to 495 of SEQ ID NO:1. Preferably, the nucleotide sequence encodes a polypeptide having phospholipase activity.

For purposes of the present invention, alignments of sequences and calculation of homology scores were done using the Lipman-Pearson Method (Lipman, D.J. and W.R. Pearson (1985) Rapid and sensitive protein similarity searches. Science 227: 1435-1441) using a PAM250 residue weight table (Dayhoff, M.O., R.M. Schwartz, and B.C. Orcutt (1978) A model of evolutionary change in proteins. In Dayhoff, M.O. (ed.), Atlas of Protein Sequence and Structure. National Biomedical Research Foundation. Washington, D.C. Vol 5. Suppl. 3: pp. 345-358) and the default settings of the MegAlign program, v4.03, in the Lasergene software package (DNASTAR Inc., 1228 South Park Street, Madison, Wisconsin 53715). The default settings are a K-tuple of 2, gap penalty of 4, and a gap length penalty of 12.

Use of phospholipase

The phospholipase of the invention can be used in various industrial application of phospholipases, e.g. as described below.

Use in baking

The phospholipase of the invention can be used in the preparation of dough, bread and cakes, e.g. to improve the elasticity of the bread or cake. Thus, the phospholipase can be used in a process for making bread, comprising adding the phospholipase to the ingredients of a dough, kneading the dough and baking the dough to make the bread. This can be done in analogy with US 4567056 or WO 99/53769.

Use in detergent

The variant may be used as a detergent additive, e.g. at a concentration (expressed as pure enzyme protein) of 0.001-10 (e.g. 0.01-1) mg per gram of detergent or 0.001-100 (e.g. 0.01-10) mg per liter of wash liquor.

The detergent composition of the invention may for example be formulated as a hand or machine laundry detergent composition including a laundry additive composition suitable for pre-treatment of stained fabrics and a rinse added fabric softener composition, or be formu-

lated as a detergent composition for use in general household hard surface cleaning operations. In a laundry detergent, the variant may be effective for the removal of fatty stains, for whiteness maintenance and for dingy cleanup. A laundry detergent composition may be formulated as described in GB 2247025, WO 9901531 or WO 9903962.

The detergent composition of the invention may particularly be formulated for hand or machine dishwashing operations, e.g. as described in GB 2,247,025 (Unilever) or WO 99/01531 (Procter & Gamble). In a dishwashing composition, the variant may be effective for removal of greasy/oily stains, for prevention of the staining /discoloration of the dishware and plastic components of the dishwasher by highly colored components and the avoidance of lime soap deposits on the dishware.

Other uses

The phospholipase of the invention can be used to improve the filterability of an aqueous solution or slurry of carbohydrate origin by treating it with the phospholipase. This is particularly applicable to a solution of slurry containing a starch hydrolyzate, especially a wheat starch hydrolyzate, since this tends to be difficult to filter and to give cloudy filtrates. The treatment can be done in analogy with EP 219,269 (CPC International).

Further, the phospholipase of the invention may be used for partial hydrolysis of phospholipids, preferably lecithin, to obtain improved phospholipid emulsifiers. This application is further described in Ullmann's Encyclopedia of Industrial Chemistry (Publisher: VCH Weinheim (1996)), JP patent 2794574, and JP-B 6-087751.

Further, the phospholipase of the invention may be used in a process for the production of an animal feed which comprises mixing the phospholipase with feed substances and at least one phospholipid. This can be done in analogy with EP 743 017.

Even further the phospholipase of the invention can be used in a process for reducing the content of phospholipid in an edible oil, comprising treating the oil with the phospholipase so as to hydrolyze a major part of the phospholipid, and separating an aqueous phase containing the hydrolyzed phospholipid from the oil. This process is applicable to the purification of any edible oil which contains phospholipid, e.g. vegetable oil such as soy bean oil, rape seed oil and sunflower oil. The phospholipase may e.g. be used in the processes described in JP-A 2-153997 and US 5264367.

Method for producing cheese

The phospholipase of the invention may be used in cheese making analogous to the use of phospholipase in cheese making described in US 6399121.

In one aspect the invention thus relates to a process for producing cheese comprising treating a dairy composition with a phospholipase of the invention and producing cheese from the dairy composition.

Another aspect of the invention relates to a process for producing cheese comprising treating a dairy composition with phospholipase and producing cheese from the dairy composition, wherein the phospholipase is selected from the group of fungal/bacterial group XIII PLA2 phospholipases. In a preferred embodiment of the invention the fungal/bacterial group XIII PLA2 is from a fungus, more preferably from a fungus belonging to the *Ascomycetes*.

A phospholipase belonging to the fungal/bacterial group XIII PLA2 may be any phospholipase belonging to this group as defined by Soragni et al., The EMBO Journal, 20 (2001), 5079-5090, and may e.g. be from the species *Tuber*, e.g. *T. borchii*, *Streptomyces*, e.g. *S. coelicor*, *Verticillium*, e.g. *V. dahliae*, *Aspergillus*, e.g. *A. oryzae*, *Neurospora*, e.g. *N. crassa*, or *Helicosporum*.

A dairy composition according to the invention may be any composition comprising milk constituents. Milk constituents may be any constituent of milk such as milk fat, milk protein, casein, whey protein, and lactose. A milk fraction may be any fraction of milk such as e.g. skim milk, butter milk, whey, cream, milk powder, whole milk powder, skim milk powder. In a preferred embodiment of the invention the dairy composition comprises milk, skim milk, butter milk, whole milk, whey, cream, or any combination thereof. In a more preferred embodiment the dairy composition consists of milk, such as skim milk, whole milk, cream, buttermilk, or any combination thereof.

The enzymatic treatment in the process of the invention may be conducted by dispersing the phospholipase into the dairy composition, and allowing the enzyme reaction to take place at an appropriate holding-time at an appropriate temperature. The treatment with phospholipase may be carried out at conditions chosen to suit the selected enzyme(s) according to principles well known in the art.

The enzymatic treatment may be conducted at any suitable pH, such as e.g., in the range 2-10, such as, at a pH of 4-9 or 5-7. In one embodiment the phospholipase treatment is conducted at 3-60°C, such as at 25-45°C (e.g., for at least 5 minutes, such as, e.g., for at least 10 minutes or at least 30 minutes, e.g., for 5-120 minutes). The phospholipase is added in a suitable amount to produce the cheese having the desired properties. Preferably, the phospholipase is added in an amount effective to decrease the oiling-off effect in cheese and/or to increase cheese yield. A suitable dosage of phospholipase will usually be in the range 0.001-0.5 mg enzyme protein per g milk fat, preferably 0.01-0.3 mg enzyme protein per g milk fat, more preferably, 0.02-0.1 mg enzyme protein per g milk fat

The cheeses produced by the process of the present invention comprise all varieties of cheese, such as, e.g. Campesino, Chester, Danbo, Drabant, Herregård, Manchego, Provo-

lone, Saint Paulin, Soft cheese, Svecia, Taleggio, White cheese, including rennet-curd cheese produced by rennet-coagulation of the cheese curd; ripened cheeses such as Cheddar, Colby, Edam, Muenster, Gruyere, Emmenthal, Camembert, Parmesan and Romano; blue cheese, such as Danish blue cheese; fresh cheeses such as Feta; acid coagulated cheeses such as cream cheese, Neufchatel, Quarg, Cottage Cheese and Queso Blanco. In a preferred embodiment the invention relates to a process for producing pasta filata cheese, such as e.g. Mozzarella and Pizza cheese. Pasta filata, or stretched curd, cheeses are normally distinguished by a unique plasticizing and kneading treatment of the fresh curd in hot water, which imparts the finished cheese its characteristic fibrous structure and melting and stretching properties, cf. e.g. "Mozzarella and Pizza cheese" by Paul S. Kindstedt, Cheese: Chemistry, physics and microbiology, Volume 2: Major Cheese groups, second edition, page 337-341, Chapman & Hall.

EXAMPLES

Material and Methods:

RA sporulation medium:

50 g succinic acid
12.1 g sodium nitrate
1 g glucose
20 ml 50x Vogel's salts (Davis, R. H. and F. J. de Serres (1970), Meth. Enzymol. 17A:79-143)
components are blended in one liter distilled water and filter sterilized

YP+2%G medium:

10 g yeast extract
20 g peptone
water to 1 l
autoclave at 121°C, 20 minutes
add 100ml 20% sterile glucose solution

Britton Robinson buffer

0.023 M phosphoric acid
0.023 M acetic acid
0.023 M boric acid

Titrated with NaOH or HCl to desired pH

Phospholipase activity (LEU assay):

Lecithin is hydrolyzed under constant pH and temperature, and the phospholipase activity is determined as the rate of titrant (0.1N NaOH) consumption during neutralization of the liberated fatty acid.

The substrate is soy lecithin (L-α-Phosphotidyl-Choline), and the conditions are pH 8.00, 40.0°C, reaction time 2 min. The unit (LEU) is defined relative to a standard.

Example 1: Cloning and expression of a phospholipase from *Fusarium venenatum* in *Aspergillus oryzae*

Cells of the *Fusarium venenatum* A3/5 (originally deposited as *Fusarium graminearum* ATCC 20334 and recently reclassified as *Fusarium venenatum* by Yoder and Christianson, 1998, Fungal Genetics and Biology 23: 62-80; and O'Donnell et al., 1998, Fungal Genetics and Biology 23: 57-67) were grown for two days in Vogel's minimal medium (Davis, R. H. and F. J. de Serres (1970), Meth. Enzymol. 17A:79-143) at 28°C in shaking culture, filtered on sterile Miracloth (Calbiochem, San Diego, California, USA), and transferred to "RA sporulation medium" in which they were incubated in shaking culture for an additional 24 hr at 28°C. Cells and spores were collected by centrifugation and lysed, and RNA was extracted and transcribed into cDNA that was cloned into pZErO-2 by the methods described in WO 00/56762. The number of independent clones in this library before amplification was 2.5x105, of which 92% contained inserts ranging in size from 550-2500 bp. Partial DNA sequences were determined for approximately 1000 randomly chosen clones and the sequences were stored in a computer database by methods described in WO 00/56762.

The nucleotide sequence of a cDNA encoding TbSP1, a phospholipase A2 from *Tuber borchii*, and the corresponding peptide translation were reported by E. Soragni et al., 2001. This translated peptide sequence was compared to translations of the *Fusarium venenatum* partial cDNA sequences using the TFASTXY program, version 3.3t08 (Pearson et al., 1997). One translated *F. venenatum* sequence was identified as having 42% identity to TbSP1 through a 125 amino acid overlap. The complete sequence of the cDNA insert of the corresponding clone, FM0700, was determined and is presented as SEQ ID NO: 1, and the peptide translated from this sequence, FvPLA2, is presented as SEQ ID NO: 2. This sequence was used to design primers for PCR amplification of the FvPLA2 encoding-gene from FM0700, with appropriate restriction sites added to the primer ends to facilitate sub-cloning of the PCR product. PCR amplification was performed using Extensor Hi-Fidelity PCR Master

Mix (ABgene, Surrey, U.K.) following the manufacturers instructions and using an annealing temperature of 52°C and an extension temperature of 60°C for 20 cycles.

The PCR fragment was restricted with BamHI and XhoI and cloned into the *Aspergillus* expression vector pMStr57 using standard techniques. The expression vector pMStr57 contains the same elements as pCaHj483 (WO 98/00529), with minor modifications made to the *Aspergillus* NA2 promoter as described for the vector pMT2188 in WO 01/12794, and has sequences for selection and propagation in *E. coli*, and selection and expression in *Aspergillus*. Specifically, selection in *Aspergillus* is facilitated by the amdS gene of *Aspergillus nidulans*, which allows the use of acetamide as a sole nitrogen source. Expression in *Aspergillus* is mediated by a modified neutral amylase II (NA2) promoter from *Aspergillus niger* which is fused to the 5' leader sequence of the triose phosphate isomerase (tpi) encoding-gene from *Aspergillus nidulans*, and the terminator from the amyloglucosidase-encoding gene from *Aspergillus niger*. The phospholipase-encoding gene of the resulting *Aspergillus* expression construct, pMStr77, was sequenced and the sequence agreed completely with that determined previously for the insert of FM0700.

The *Aspergillus oryzae* strain BECh2 (WO 00/39322) was transformed with pMStr77 using standard techniques (T. Christensen et al., 1988). Transformants were cultured in YP+2%G medium shaken at 275 RPM at 30°C and expression of FvPLA2 was monitored by SDS-PAGE.

Example 2: Purification and sequence comparison

FvPLA2 from the fermentation of example 1 was purified by ion exchange chromatography on a SP-sepharose column equilibrated with 50 mM Acetate-buffer pH 4.7, and eluted with 1M NaCl pH 4.7. Fractions were analyzed on SDS-PAGE, and fractions containing a 14 kDa protein were pooled. The identity of the pure protein was confirmed by determining the the N-terminal sequence, which was identical to the sequence from amino acid (aa) 29-40 of SEQ ID NO:2. Additionally, the mass of the peptide was determined by mass spectral analysis, because the apparent size estimated from SDS-PAGE, 14 kDa, is smaller than that of the peptide predicted by processing the theoretical peptide in SEQ ID NO:2. The mass of the purified, active FvPLA2 was found to be 13336 Da. This molecular mass indicates additional processing at the C-terminus, and is consistent with a cleavage between amino acids 149 and 150 in SEQ ID NO:2, as the peptide sequence from amino acid 29 to 149 has a theoretical mass of 13335,86 Da.

A comparison of the mature processed peptide (amino acids 29-149 of SEQ ID NO:2) with known sequences showed that the closest prior-art sequence was a phospholipase from *Verticillium dahliae* translated from Unisequence ID: VD0100C34 from the COGEME Phytopathogenic Fungi and Oomycete EST Database Version 1.2 (http://cogeme.ex.ac.uk/) (Soanes

et al. (2002) Genomics of phytopathogenic fungi and the development of bioinformatic resources. Mol Plant Microbe Interact. 15(5):421-7). The processing of the partial peptide predicted from the *V. dahliae* sequence was estimated by comparison to the found processing for FvPLA2. The identity between amino acids 29 to 149 of SEQ ID NO: 2 and the estimated sequence of the mature peptide of the *V. dahliae* phospholipase was calculated to be 77%.

Example 3: Physical properties

Catalytic activity

Phospholipase activity as a function of enzyme concentration was determined in the LEU assay. Results are shown in table 1.

Table 1

Enzyme conc. (µg/ml)	LEU (µeq NaOH/min)
71.1	14.0
53.3	12.7
21.3	10.6
10.7	7.4
5.3	5.6
2.7	4.1

Temperature profile

The enzyme activity as a function of temperature was determined for an enzyme solution with a concentration of 5.3 µg/ml. Other conditions as in the LEU assay. Results are shown in table 2.

Table 2

Temperature (°C)	LEU (µeq NaOH/min)

25	3.10
35	4.87
40	5.41
45	6.97
50	7.86
55	9.03
60	8.27
65	6.90

pH stability

The enzyme was diluted in a Britton Robinson buffer at the specified pH for 30 min at 30°C. After further dilution in water catalytic activity was measured in the LEU assay. Results are shown in table 3.

Table 3

pH	LEU (µeq NaOH/min)
2	3.78
3	5.11
4	5.60
5	5.49
6	5.37
7	5.61
8	5.52
9	5.64
10	5.50
11	5.21

Thermo stability

The enzyme was diluted in Britton Robinson buffer at pH 3 and 10 respectively, and at pH 7 with 30% sorbitol. After incubation at the specified temperature for 30 minutes, the solution was cooled to the reaction temperature and assayed in the LEU assay. The results are shown in table 4; activities are given relative to the highest measured activity.

Table 4. Relative activity (%) as a function of pH and temperature

Temperature (°C)	pH 3	pH 10	pH7/30% sorbitol
30	100%	100%	87%
40	95%	92%	100%
50	16%	14%	68%
60	1%	0%	2%

Example 3: Cheese making

Pasteurized, non-homogenized cream (North Carolina State University Dairy Plant) was used to standardize five hundred grams pasteurized, non-homogenized skim milk (North Carolina State University Dairy Plant) to 3.5% fat thus producing full fat mozzarella cheese.

The cheese milk for each experiment was treated with either the *F. venenatum* phospholipase (FvPLA2) prepared according to example 1, or of the commercial phospholipase Lecitase® 10L (Novozymes A/S, Bagsværd, Denmark), and placed in a 35°C water bath until equilibrated to that temperature. The initial pH of the cheese milk was taken and 0.01% (w/w) of starter culture at was added.

pH was monitored until a pH of 6.4 was reached. 250 µl rennet (Novozym 89L) was diluted to in 9 ml total solution with deionized water, one ml of this solution was added to the cheese milk and the cheese milk was stirred vigorously for 3 minutes. The stir bar was removed and the rennetted milk was allowed to sit at 35°C.

After the above treatments, curd was ready to cut when a spatula was inserted and sharp edges were seen. The cheese was cut by pushing the cutter down and while holding the beaker quickly turning the cutter and finally pulling the cutter up. The curd was allowed to rest 5 minutes then stirred gently with spoon. Temperature was raised to 41°C with intermittent gentle agitation for ~ 45 min or until the pH dropped to 6.0-5.9. The curd was drained using cheesecloth then replaced in the beaker and kept at 41°C in water bath while pouring off whey as needed.

When the curd reached pH 5.3, the stainless steel bowl with the curd in it was flooded in a water bath at 69°C for 5 minutes then hand stretched. Curd was tempered in cold icewater for 30 minutes. The cheese curd was dried out with paper towel, weighed and refrigerated overnight.

Control cheese making experiments were made from the same batch of milk following the same procedures except that no phospholipase was added.

Actual cheese yield was calculated as the weight of cheese after stretching relative to the total weight of cheese milk.

Moisture adjusted cheese yield was expressed as the actual yield adjusted to standard constant level of moisture. Moisture adjusted yield was calculated by multiplying the actual yield and the ratio of actual moisture content to standard moisture, according to the following formula:

$$Y_{adj} = Y_{act} \times (1 - M_{act}) / (1 - M_{std})$$

where Y_{adj} = moisture adjusted cheese yield, Y_{act} = actual cheese yield, M_{act} = actual moisture fraction & M_{std} = standard moisture fraction (0.48).

The moisture adjusted cheese yield of all experiments and controls are shown in table 5

Table 5

Treatment	Phospholipase mg enzyme protein/g fat	Moisture adjusted cheese yield	Yield increase compared to control
Control	0	11.70	
FvPLA2	0.071	11.95	2.1%
Control	0	11.50	
FvPLA2	0.071	11.83	2.8%
Control	0	9.22	
Lecitase® 10L	0.18	9.48	2.7%
Control	0	9.62	
Lecitase® 10L	0.18	9.90	2.8%

CLAIMS

1. A phospholipase which comprises:

a) a polypeptide encoded by the phospholipase encoding part of the DNA sequence cloned into a plasmid present in *Escherichia coli* deposit number DSM 15442;

or

b) a polypeptide comprising the amino acid sequence of amino acids 29 to 149 of SEQ ID NO: 2, or an amino acid sequence which can be obtained therefrom by substitution, deletion, and/or insertion of one or more amino acids;

or

c) an analogue of the polypeptide defined in (a) or (b) which:

i) has at least 80 % homology with said polypeptide, or

ii) is immunologically reactive with an antibody raised against said polypeptide in purified form, or

iii) is an allelic variant of said polypeptide;

or

d) a polypeptide which is encoded by a nucleic acid sequence which hybridizes under low stringency conditions with a complementary strand of the nucleic acid sequence of nucleic acids 133 to 495 SEQ ID NO: 1 encoding the mature polypeptide or a subsequence thereof having at least 100 nucleotides.

2. The phospholipase of claim 1 which is native to a strain of *Fusarium*, particularly *F. venenatum*.

3. A nucleic acid sequence comprising a nucleic acid sequence which encodes the phospholipase of claims 1 or 2.

4. A nucleic acid sequence which comprises:

a) the partial DNA sequence encoding a mature phospholipase cloned into a plasmid present in *Escherichia coli* DSM 15442,

b) the partial DNA sequence encoding a mature phospholipase of nucleic acids 133 to 495 of SEQ ID NO: 1,

c) an analogue of the sequence defined in a) or b) which encodes a phospholipase and

i) has at least 80 % homology with said DNA sequence, or

ii) hybridizes at high stringency with a complementary strand of said DNA sequence or a subsequence thereof having at least 100 nucleotides,

iii) is an allelic variant thereof, or

d) a complementary strand of a), b) or c).

5. A nucleic acid construct comprising the nucleic acid sequence of claims 3 or 4 operably linked to one or more control sequences capable of directing the expression of the phospholipase in a suitable expression host.

6. A recombinant expression vector comprising the nucleic acid construct of claim 5, a promoter, and transcriptional and translational stop signals.

7. A recombinant host cell comprising the nucleic acid construct of claim 6.

8. A method for producing a phospholipase comprising cultivating the host cell of claim 7 under conditions conducive to production of the phospholipase, and recovering the phospholipase.

9. A method for preparing a dough or a baked product made from the dough, comprising adding the phospholipase of claim 1 to the dough.

10. A dough composition comprising the phospholipase of claim 1.

11. A detergent composition comprising a surfactant and the phospholipase of claim 1.

12. A process for reducing the content of phosphorus in a vegetable oil, comprising contacting the oil with the phospholipase of claim 1 in the presence of water, and then separating an aqueous phase from the oil.

13. A process for producing cheese comprising treating a dairy composition with a phospholipase and producing cheese from the dairy composition, wherein the phospholipase is the phospholipase of claim 1.

14. A process for producing cheese comprising treating a dairy composition with a phospholipase and producing cheese from the dairy composition, wherein the phospholipase is selected from the group of fungal/bacterial group XIII PLA2 phospholipases.

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